BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 3/18, B01D 15/08 — A1

(11) International Publication Number: WO 93/05068

(43) International Publication Date: 18 March 1993 (18.03.93)

(21) International Application Number: PCT/SE92/00610

(22) International Filing Date: 4 September 1992 (04.09.92)

(30) Priority data:
9102622-9 6 September 1991 (06.09.91) SE

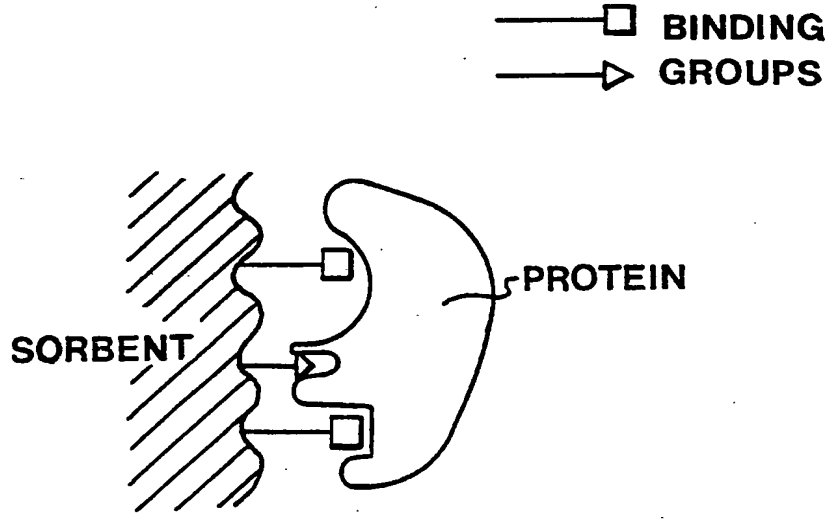
(71)(72) Applicants and Inventors: GLAD, Magnus [SE/SE]; Nyckelkroken 64, S-222 47 Lund (SE). KEMPE, Maria [SE/SE]; Knut den Stores gata 1 C, S-222 21 Lund (SE). MOSBACH, Klaus [SE/SE]; Lackalänga 31, P1 5548, S-244 94 Furulund (SE).

(74) Agent: AWAPATENT AB; Box 5117, S-200 71 Malmö (SE).

(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.
In English translation (filed in Swedish).

(54) Title: SELECTIVE AFFINITY MATERIAL, PREPARATION THEREOF BY MOLECULAR IMPRINTING, AND USE OF THE SAME



(57) Abstract

A selective adsorption material, especially suitable for adsorption of biological macromolecules, is described. The adsorption material comprises a matrix with immobilised ligands, localised to selectively adsorb a predetermined molecule. A process for preparing a selective adsorption material, especially suitable for adsorption of biological macromolecules, is also described. The process is characterised in that a print molecule having at least two separate binding sites is bonded to the corresponding, at least two immobilisable ligands, the ligands are immobilised, and subsequently the print molecule is removed. The selective adsorption material can be used for purification or analysis, especially of biological macromolecules.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MN	Mongolia
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Faso	GN	Guinea	NO	Norway
BG	Bulgaria	GR	Greece	NZ	New Zealand
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	PT	Portugal
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovak Republic
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CS	Czechoslovakia	LU	Luxembourg	SU	Soviet Union
CZ	Czech Republic	MC	Monaco	TD	Chad
DE	Germany	MG	Madagascar	TG	Togo
DK	Denmark	ML	Mali	UA	Ukraine
ES	Spain			US	United States of America

SELECTIVE AFFINITY MATERIAL, PREPARATION THEREOF BY MOLECULAR IMPRINTING, AND USE OF THE SAME.

The invention relates to a selective adsorption material, especially suitable for adsorption of biological macromolecules, a process for preparing this selective adsorption material, and the use thereof for purification and analysis, especially of biological macromolecules.

It is known to prepare homogeneous gels while using the so-called molecular imprinting technique by making imprints of dyes (R. Arshady, K. Mosbach; Makromol. Chemie, 182 (1981) 687) and amino acid derivatives (L. Andersson, B. Sellergren, K. Mosbach, Tetrahedron Lett. 25 (1984) 5211). Merely non-covalent bonding between a "print molecule" and monomers is used. After polymerisation of the monomers and removal of the print molecule, a selective polymer is obtained, based on binding groups correctly immobilised in space and present in cavities which have the shape of a mould of the print molecule. A summary of this technique is given by B. Ekberg, K. Mosbach, in Trends Biotechnol., 7 (1989) 92.

It is known to make imprints of carbohydrate derivatives substituted with covalently, but reversibly bonded vinylphenyl boric acid groups, so-called boronate esters, which after polymerisation have permitted hydrolysis and binding of a new print molecule (G. Wulff, ACS Symp. Series, 308 (1986) 186). Decisive of the selectivity of the thus prepared polymers are correctly positioned binding boronate groups and a well-shaped cavity. The drawback of this system is that a complicated chemical synthesis is necessary.

Prearranged boronate groups have also been used for making imprints of glycoprotein ((Transferrin, M. Glad, O. Norrlöw, B. Sellergren, N. Siegbahn, K. Mosbach, J. Chromatogr., 347 (1985) 11) and bis-nucleotides (O. Norrlöw, M. O. Månsson, K. Mosbach, J. Chromatogr.,

396 (1987) 374) on silica. In this context, use has been made of a mixture of non-covalently bonding organic silanes and boronate silane, which has been caused to interact with a print molecule before polymerisation on the surface. The effect of recognition seems mainly to depend on the fact that the boronate groups are correctly spaced from each other to be able to interact effectively with the print molecules. Transferrin has a total of four sialic acid groups and bis-NAD has four riboses which form boronate esters.

It is also known to use, in affinity chromatography methods, adsorption material having specific ligands, but these are randomly localised on the sorbent and may therefore yield poor selectivety. Furthermore, there will be a large number of unused ligands, which is uneconomical from the industrial point of view.

Immobilised phenyl boric acid has been used to separate carbohydrate derivatives (H.L. Weith, J.L. Wiebers, P.T. Gilham, Biochemistry, 91 (1970) 4) and also to separate glycosylised hemoglobin and other glycoproteins (P.D.G. Dean, P.J. Brown, V. Bouriotis, US Patent Specification 4,269,605 (1981).

For purification and analysis, especially of biological macromolecules, selectivity is most important. It is frequently necessary to distinguish a single component in a mixture of, maybe, several thousand components. The known adsorption materials are in most cases not sufficiently selective and cause non-specific binding, which may completely destroy the result of an analysis.

Modern biotechnical production and analysis as well as a large amount of medical diagnostics are based on selective binding. The methods known so far are also for these purposes not sufficiently selective and/or sufficiently simple to be economically useful on an industrial scale. Therefore there is a need of an adsorption material having very high selectivity and low non-specific binding, which may be prepared on a large scale and at low cost.

According to the invention, a selective adsorption material is provided, which is especially suitable for adsorption of biological macromolecules, and which is characterised in that it comprises immobilised ligands which have been localised to selectively adsorb a predetermined molecule by their first being bound to a print molecule which has then been removed after the immobilisation of the ligand.

The invention also comprises a process for preparing a selective adsorption material, especially suitable for adsorption of biological macromolecules, characterised in that a print molecule having at least two separate binding sites is bonded to the corresponding, at least two immobilisable ligands, that the ligands are immobilised, and that subsequently the print molecule is removed.

Finally the invention relates to the use of the selective adsorption material for purification or analysis, especially of biological macromolecules.

After prebonding to the print molecule and the subsequent immobilisation, the binding groups (ligands) will be bonded preferably to the surface of a matrix. The correct localisation of ligands on two dimensions results in increased selectivity by the print molecule having at least two separate binding sites. The corresponding, at least two immobilised ligands will thus be correctly spaced apart. This results in optimal binding of the target molecule in the subsequent adsorption.

As is evident from that stated above, the invention thus relates to a form of directed immobilisation of affinity ligands or other (chemical) ligands. This directed immobilisation is achieved by means of a prebinding method. The advantage as compared to randomly immobilised binding groups is that the print molecule is bonded more effectively and with greater selectivity. At the same time, all ligands may be utilised, thereby avoiding an excess thereof, which otherwise may easily result in non-specific bonding which in turn results in a lower yield.

Ligands which are useful for the invention should carry groups that can be bonded to a print molecule, and groups which can be used for immobilisation of the ligands. One type of suitable ligands is monomers which can be immobilised by polymerisation. Examples of groups that can be bonded to a print molecule are given in Table 1.

Table 1

Group binding to print molecule on ligand/monomer	Binding group on print molecule
Imidazole-metal	Histidine, tryptophane, thiol
Iminodiacetic acid-metal	" " "
Carboxyl	Amine
Amine	Carboxyl
Carboxyl	Carboxyl, amide (hydrogen bonding)
Boronate	Carbohydrate, diol
Thiol	Thiol
Aldehyde	Amine

Boronate, thiol and aldehyde are bonded covalently, whereas the other groups are bonded non-covalently to the print molecule.

Among groups that may be used for immobilisation, mention can be made of silanes which bind silanol and hydroxyl groups, and vinyl, acryl and methacryl which polymerise on a matrix.

Other ligands/monomers that may be used are:
Acryloylhistamine: H. Morawetz, W.R. Song, JACS, 88 (1966) 571.
Boronate silane: M. Glad, O. Norrlöw, B. Sellergren, N. Siegbahn, K. Mosbach, J. Chromatogr., 347 (1985) 11.
IDA-silane: A. Figueroa, C. Corradini, B. Feibush, B.L. Karger, J. Chromatogr., 371 (1986) 335.

Moreover, different types of ligands may be used simultaneously in one preparation. For example, use can be made of both boronate binding and metal interaction on the

same print molecule if this is e.g. a glycoprotein with surface-bound histidines.

As print molecule (imprinting molecule) for prearrangement of the ligands, the invention preferably uses biological macromolecules, e.g. enzymes, antibodies, glycoproteins or other proteins, nucleic acids, polysaccharides. The molecular imprinting is preferably carried out in aqueous media which are the normal surroundings of these molecules. It has also become apparent that larger or smaller amounts of organic solvents which are miscible with water, such as DMF, DMSO, formamide (M. Ståhl, M.O. Månsson, K. Mosbach, Biotechn. Lett., 12 (1990) 161; N. Chang, S.J. Hen, A.M. Klibanov, BBRC, 176 (1991) 1462), may be added. In some cases, also pure DMSO can be used, in which protein and a monomer mixture (the ligands) are dissolved. A polymerised product is obtained, having a sufficient amount of correctly positioned, binding groups to provide a certain selectivity.

Matrix materials which are useful according to the invention must contain surface-bound reactive groups which can react with and covalently bind prearranged ligands. The matrices may be solid or in the form of gels and comprise particles, chips, electrodes, gel etc. Suitable matrix materials are silica (particles, chips etc.), glass (particles, electrodes etc.), biological polymers (agarose, dextran, gelatin etc.) and synthetic polymers (polyvinyl alcohol, TRIM (P. Reinholdsson, T. Hargitai, B. Törnell, R. Isaksson, Angew. Macromol. Chem., 1991, in press) etc.). When the matrix consists of a particulate solid phase, the surface is to be found both on the outside of the particles and on the surface of the pores thereof. In most cases, the latter surface is many times larger than the former.

Reactive groups on the matrix material can be acrylate, methacrylate, vinyl, hydroxyl or silanol.

If the matrix material contains no suitable reactive groups, it may be derivatised in advance with suitable groups, such as methacryl groups, before immobilising the ligands on the matrix.

The difference between a prior art adsorption material and the invention is schematically shown in the accompanying drawing in which:

Fig. 1 is a schematic sketch of a prior art sorbent with randomly positioned, binding groups, e.g. boronate, immobilised metal; and

Fig. 2 shows a sorbent according to the invention, with specifically localised, binding groups.

The invention will now be described in more detail by means of the following, non-restrictive Example.

Example

Methacrylate silica

3-methacryloyloxypropyltrimethoxysilane (2.0 g, 8.1 mmol, Fluka) was mixed with 100 ml of water and dissolved during powerful stirring by means of a magnetic stirrer at 22°C for 4 h. Porous silica (5.0 g, "Lichrospher Si 300", Merck) was suspended in 20 ml of water and treated with vacuum and ultrasonics, thereby producing pores free from air. Subsequently, the silane solution was added, and the silanisation was allowed to proceed for 4 h at 60°C in a round-bottomed flask placed in a water bath and fitted with a "Teflon®"-coated blade mixer. The derivatised silica was washed on a glass filter with water and methanol and then air-dried on the filter.

Titration with bromine water (according to Glad et al, J. Chromatogr., 347 (1985) 11) yielded 335 µmol of methacrylate groups per g of silica product. A carbon analysis yielded 620 µmol of silane per g of silica product, which indicates that more than half of the groups are still reactive to subsequent immobilisation.

Preparation

Mixtures according to the Table below with RNase B and STI (Soybean trypsine inhibitor), respectively, as print molecule.

	mg	µmol
Methacrylate-silica	800	268
Vinyl imidazole[1)]	0.39	4.14
Acrylphenyl boric acid	1.59	8.32
Acrylamide	100	1410
PDA (piperazine diacrylamide)	50	257
TEMED	5	
$ZnCl_2$	0.64	4.70
Water/DMF (7/3, w/w)	2.5 ml	
Ammonium persulphate		
alt. A) RNase B (molecular weight 14700)	10	0.73
alt. B) STI (molecular weight 20100)	10	0.53

1) ref. C.G. Overberger, N. Vorchheimer, JACS, 85 (1963) 951

After mixing the respective print molecule with all the other ingredients in a total of 2.5 ml of water/DMF, the samples were cooled, and then N_2 gas was conducted through the mixture. After about 30 s, the mixture began to solidify. The preparation was allowed to stand for 1 h, and subsequently the substituted silica particles were washed on a glass filter with water/DMF. Small polymer particles were removed by sedimentation, and the remaining particles were packed in steel columns (5 x 0.5 cm). When injecting RNase B on preparation A, the elution was delayed as compared with preparation B.

As an alternative to vinyl imidazole, vinyl benzyl iminodiacetic acid can be used (L.R. Morris, R.A. Mock, C.A. Marshall, J.H. Howe, JACS, 81 (1959) 377). These bind metal ions, e.g. Zn^{2+}, Cu^{2+}, which have been used for a long time in so-called immobilised metal affinity chromatography (J. Porath, J. Carlsson, I. Olsson, G. Belfrage, Nature, 258 (1975) 598).

CLAIMS

1. Selective adsorption material, especially suitable for adsorption of biological macromolecules, c h a r a c t e r i s e d by immobilised ligands which have been localised to selectively adsorb a predetermined molecule by their first being bound to a print molecule which has then been removed after the immobilisation of the ligands.

2. Adsorption material as claimed in claim 1, c h a r a c t e r i s e d in that the ligands are polymerisable monomers which have been immobilsed by polymerisation.

3. Adsorption material as claimed in claim 1, c h a r a c t e r i s e d in that the ligands are immobilised by covalent bonding to a matrix having surface-bound groups which are reactive with the ligands.

4. Adsorption material as claimed in claim 1, c h a r a c t e r i s e d in that the ligands are immobilised in a polymer network which is polymerised or bonded to a matrix.

5. Adsorption material as claimed in claim 3 or 4, c h a r a c t e r i s e d in that the matrix is selected among silica, glass, biological polymers, synthetic polymers and mixtures thereof.

6. Adsorption material as claimed in one or more of the preceding claims, c h a r a c t e r i s e d in that the ligands have been bound to the print molecule via one or more of the groups imidazole-metal, iminodiacetic acid-metal, carboxyl, amine, boronate, thiol and aldehyde on the ligands.

7. Adsorption material as claimed in claim 6, c h a r a c t e r i s e d in that the ligands have been bound to the print molecule via the groups imidazole-metal and/or iminodiacetic acid-metal on the ligands.

8. Process for preparing a selective adsorption material, especially suitable for adsorption of biological macromolecules, c h a r a c t e r i s e d in that a print molecule having at least two separate binding sites is bonded to the corresponding, at least two immobilisable ligands, whereupon the ligands are immobilised, and subsequently the print molecule is removed.

9. Process as claimed in claim 8, c h a r a c t e r i s e d in that the ligands are polymerisable monomers and are immobilised by direct polymerisation.

10. Process as claimed in claim 8, c h a r a c t e r i s e d in that the immobilisation of the ligands is effected by covalent bonding to a matrix having surface-bound groups which are reactive with the ligands.

11. Process as claimed in claim 10, c h a r a c t e r i s e d in that the matrix is, by prederivatisation, provided with groups which can react with the ligands.

12. Process as claimed in claim 8, c h a r a c t e r i s e d in that the ligands are immobilised by binding in a polymer network which is polymerised or bonded to a matrix.

13. Process as claimed in one or more of claims 10-12, c h a r a c t e r i s e d in that the matrix is selected among silica, glass, biological polymers, synthetic polymers and mixtures thereof.

14. Process as claimed in one or more of claims 8-13, c h a r a c t e r i s e d in that the ligands are bound to the print molecule via one or more of the groups imidazole-metal, iminodiacetic acid-metal, carboxyl, amine, boronate, thiol and aldehyde on the ligands.

15. Process as claimed in claim 14, c h a r a c t e r i s e d in that the ligands are bound to the print molecule via the groups imidazole-metal and/or iminodiacetic acid-metal on the ligands.

16. Use of the selective adsorption material as claimed in one or more of claims 1-7, for purification or analysis, especially of biological macromolecules.

FIG.1

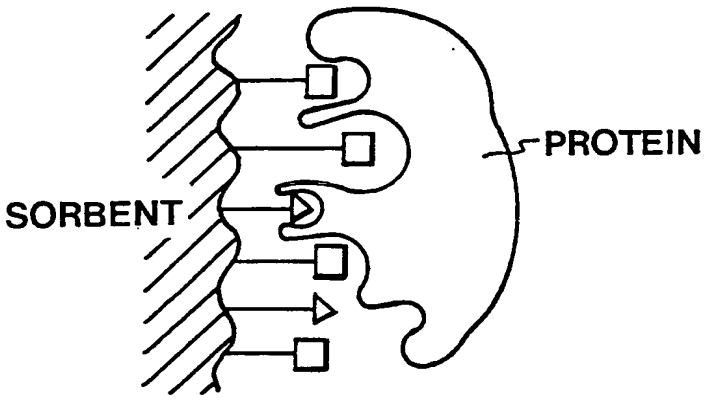
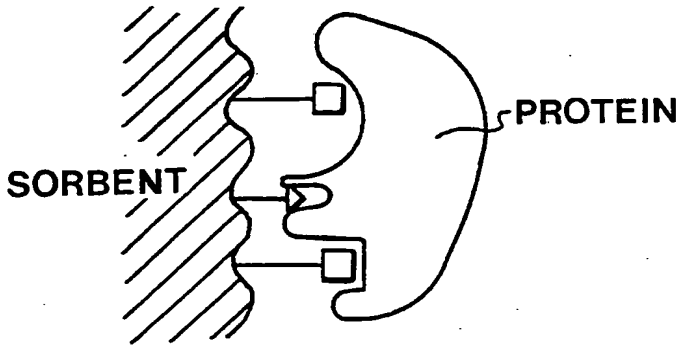


FIG.2



INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 92/00610

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 3/18, B 01 D 15/08

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 K; B 01 D

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	TIBTECH, Vol. 7, 1989 Björn Ekberg et al: "Molecular imprinting: a technique for producing specific separation materials", pp 92-96 --	1-5, 16
X	JOURNAL OF CHROMATOGRAPHY, Vol. 347, 1985 Magnus Glad et al: "Use of silane monomers for molecular imprinting and enzyme entrapment in polysiloxane-coated porous silica", pp 11-23, see especially pages 20-21 --	1-16

---

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th December 1992	16 -12- 1992
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Mikael G:son Bergstrand

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/SE 92/00610

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 30/10/92
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date